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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR § 1.53(c).

For: **HUMANIZED IGG1 ANTIBODIES DERIVED FROM CHIMPANZEE FABS THAT CROSS-NEUTRALIZE DENGUE TYPE 1 AND TYPE 2 VIRUSES EFFICIENTLY**

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Enclosed are:

(X) Specification in 2 parts.

1. Humanized IgG1 antibodies derived from chimpanzee fabs that cross-neutralize dengue type 1 and type 2 viruses efficiently in 24 pages with 3 pages of tables and 5 pages of drawings.

2. Epitope determinants of a chimpanzee fab antibody that efficiently cross-neutralizes dengue type 1 and type 2 viruses map to inside and in close proximity to the fusion loop of the dengue type 2 virus envelope glycoprotein in 25 pages with 3 pages of tables and 8 pages of drawings.

(X) A check in the amount of $160 to cover the filing fee is enclosed.

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(X) Yes. The name of the U.S. Government agency and the Government contract number are: National Institutes of Health.

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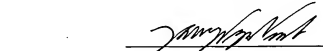

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Humanized IgG1 Antibodies Derived from Chimpanzee Fabs That Cross-Neutralize Dengue Type 1 and Type 2 Viruses Efficiently

SUMMARY

Passive immunization using monoclonal antibodies from humans or non-human primates represents an attractive alternative to vaccines for prevention of illness caused by dengue viruses and other flaviviruses, including the West Nile virus. Previously, we described repertoire cloning to recover Fab fragments from bone marrow mRNA of chimpanzees infected with all four dengue virus serotypes (DENV-1 to DENV-4). In that study we recovered and characterized a humanized IgG1 antibody that efficiently neutralized DENV-4. In this study, the phage library constructed from the chimpanzees was used to recover Fab antibodies against the other three dengue virus serotypes. Serotype-specific neutralizing Fabs were not identified. Instead, we recovered dengue virus-neutralizing Fabs that were cross-reactive with all four dengue serotypes. Three of the Fabs competed with each other for binding to DENV-1 and DENV-2, although each of these Fabs contained a distinct CDR3-H sequence. Fabs that shared an identical or nearly identical CDR3-H sequence cross-neutralized DENV-1 and DENV-2 at a similar high 50% plaque reduction ($PRNT_{50}$) titer, ranging from 0.26 to 1.33 μg/ml, and neutralized DENV-3 and DENV-4 but at a titer 10-20 fold lower. One of these Fabs, 1A5, also neutralized the West Nile virus most efficiently among other flaviviruses tested. Fab 1A5 was converted to a full-length antibody in combination with human sequences for production in mammalian CHO cells. Humanized IgG1 1A5 proved to be as efficient as Fab 1A5 for cross-neutralization of DENV-1 and DENV-2 at a titer of 0.48 and 0.95 μg/ml, respectively. IgG1 1A5 also neutralized DENV-3, DENV-4 and the West Nile virus at a $PRNT_{50}$ titer of approximately 3.2-4.2 μg/ml. This humanized antibody represents an attractive candidate for further development of immunoprophylaxis against dengue and perhaps other flaviviruses-associated diseases.

INTRODUCTION

The four dengue virus serotypes (DENV-1 to DENV-4) and several other arthropod-borne flaviviruses, including tick-borne encephalitis virus (TBEV), and yellow fever virus (YFV), Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV) and West Nile virus (WNV) are important human pathogens. Currently, dengue viruses are the most important in terms of morbidity and geographic distribution (15, 24). Patients with dengue usually develop fever, rash, and joint pain, and the disease is self-limited. Occasionally, more severe forms of disease, known as dengue haemorrhagic fever and dengue haemorrhagic shock syndrome (DHF/DSS), also occur especially in the dengue endemic regions of Southeast Asia and more recently of Central and South America. It is estimated that 50-100 million dengue infections and several hundred thousand cases of DHF occur every year. *Aedes aegypti* and *A. albopictus* mosquitos are the principal vectors for human-to-human transmission of dengue viruses. Control of dengue epidemics by spraying of insecticides to reduce the vector mosquito population has proven to be rather ineffective. *Aedes* mosquito species are also responsible for transmission of WNV, which emerged for the first time in New York in 1999 (20). Since that time the virus has spread widely to most of the continental U. S. There were several thousand reported WNV infections with mortality of two hundred in 2002 (29). Prevention of WNV infections has become an important public health issue in the U. S. and many other countries.

Dengue infection is thought to induce a life-long immunity against the same serotype of virus. Cross-protection against other dengue virus serotypes (heterotypic immunity) in humans is brief, lasting only 2-9 months (33). Concurrent or sequential infections with different dengue virus serotypes are common (16, 19, 40). Epidemiological data suggest that a subsequent infection with a dengue virus serotype different from the serotype of the previous infection is more frequently associated with severe dengue illness than is the primary dengue infection. This observation has led to the hypothesis that immunopathological mechanisms involving the activities of dengue virus-specific antibodies or cytotoxic T cells contribute to dengue severity (17, 18). However, evidence also indicates that dengue virulence could be in part due to a virus factor, such as

replication capacity (32, 39). In order to better protect against dengue infection and to minimize the risk of severe dengue, the current immunization strategy favors the use of a tetravalent vaccine against all four dengue serotypes. However, development of a safe and effective vaccine against dengue has been elusive.

Previously, we described an alternative strategy for the prevention of dengue fever by passive immunization with humanized antibodies (23). Repertoire cloning was employed to identify Fab antibody fragments from chimpanzees infected with all four dengue virus serotypes. One of these Fabs, 5H2, efficiently neutralized DENV-4 and was subsequently converted to a full-length IgG antibody containing human IgG sequences. Humanized antibody IgG 5H2 was produced in mammalian CHO cells and shown to neutralize DENV-4 at a 50% plaque reduction ($PRNT_{50}$) titer of 0.03-0.05 μg/ml by a plaque reduction neutralization test (PRNT). This success prompted us to employ the phage library constructed from the chimpanzee infected with multiple dengue virus serotypes in an effort to recover Fab fragments against the other three dengue virus serotypes. In this study, we describe identification of Fab fragments that are broadly cross-reactive with all four dengue viruses as well as with other major insect-borne flaviviruses. Several of these Fabs were shown to cross-neutralize DENV-1 and DENV-2 at a similar high titer and DENV-3 or DENV-4 at a reduced titer. A full-length humanized IgG1 antibody, designated IgG 1A5, was produced by combining Fab 1A5 with human IgG1 sequences. Humanized IgG1 1A5 antibody, like Fab 1A5, efficiently neutralized DENV-1 and DENV-2, but less efficiently neutralized DENV-3 and DENV-4 as well as other flaviviruses. Humanized antibody IgG1 1A5 represents an attractive candidate for further development of immunoprophylaxis against dengue viruses.

Materials and Methods

Inoculation of chimpanzees with multiple dengue virus serotypes and preparation of lymphocytes from bone marrow.

As described previously, two chimpanzees (#1616 and #1618) that had been intra-hepatically transfected with infectious RNA transcripts of a full-length DENV-4 cDNA clone (23) were infected subscutaneously (sc) nine-and-half months later with a mixture of DENV-1 (Western Pacific strain), DENV-2 (New Guinea C, prototype strain) and

DENV-3 (strain H87), each at 10^6 plaque forming units (pfu), diluted in minimal essential medium (MEM) plus 0.25% human serum albumin. Twelve weeks after infection with the multiple dengue virus serotypes, bone marrow was aspirated from each chimpanzee and the lymphocytes were prepared by centrifugation on a Ficoll-Paque gradient.

Construction of γ1/κ chimpanzee Fab antibody library.

Repertoire cloning of chimpanzee Fab fragments was described earlier (23). Briefly, approximately $3x10^7$ bone marrow lymphocytes from chimpanzee 1618, which developed higher neutralizing antibody titers against DENV-1, DENV-2 and DENV-3 than did chimpanzee #1616, were used for phage library construction. Total RNA from lymphocytes was extracted using the RNA Extraction kit (Stratagene, La Jolla, CA) and reverse-transcribed with oligo dT as primer using the ThermoScript RT-PCR system (Invitrogen). Chimpanzee V_L-C_L DNA sequences were amplified by PCR using seven pairs of human κ light chain family-specific primers and a constant domain 3' primer using AmpliTaq DNA polymerase (Perkin Elmers) (2, 13, 31, 35). Chimpanzee V_H-C_{H1} DNA sequences were similarly amplified using nine human γ1 heavy chain family-specific 5' primers and a chimpanzee γ1 specific 3' primer across the constant domain 1-hinge junction (13, 35).

Pooled κ light chain DNA fragments were digested with SacI and XbaI and then cloned into the pComb 3H vector by electroporation of eletrocompetent *E. coli* XL-1 Blue (Stratagene). The recombinant plasmid was used for cloning of the pooled γ1 heavy chain DNA fragments at the XhoI and SpeI sites. A library size of $2\text{-}4x10^8$ colonies of transformed *E. coli* was obtained at each cloning.

Preparation of dengue viruses from infected mosquito C6/36 cells.

Mosquito C6/36 cells were grown in MEM supplemented with 10% fetal bovine serum (FBS) plus gentamycin and fungizone. Confluent cells were infected with DENV-1, DENV-2, DENV-3 or DENV-4 of the strain indicated above, each at 0.1 multiple of infection (moi) in MEM containing 2% FBS. DENV-1, prototype Hawaii strain, kindly supplied by L. Rosen and DENV-2, New Guinea B strain, kindly supplied by W.

Schlesinger, were also used. Infected cells were placed in serum-free medium (VP-SFM, Gibco Corp) one day after infection and incubated at 28 C. The culture medium was harvested on days 6, 8, and 10 after infection and fresh serum-free medium was added after each harvest. The virus titer in the medium was determined by a focus assay on Vero cells and the medium was kept refrigerated for use as panning antigen and for ELISA and neutralization assays.

Preparation of WNV/DENV-4 chimera, JEV, and LGTV.

Vero cells were grown in MEM supplemented with 10% FBS plus gentamycin and fungizone at 37 C. Confluent Vero cell monolayers were infected with 1 moi of Langat virus strain TP 21(LGTV), kindly supplied by R. Shope, or WNV/DENV-4 chimera, kindly supplied by A. Pletnev and the infected cells were placed in MEM containing 2% fetal calf serum. JEV vaccine strain SA14-14-2, kindly supplied by K. Eckels and R. Putnak, was also propagated in Vero cells. The culture medium was harvested 7 days after infection and titered by focus assay on Vero cells. For use as ELISA antigens, LGTV, JEV and WNV/DENV-4 were grown in serum-free medium as described above. For neutralization assays, each of the above virus stocks was prepared in MEM containing 20% FBS and frozen until use.

Panning of phage library using DENV-1, DENV-2 or DENV-3 as antigens.

The pComb H DNA library that contained the V_L-C_L and V_H-C_{H1} inserts constructed earlier was again used for phage preparation. Identification of Fabs that were recovered from separate pannings against DENV-1, DENV-2 or DENV-3 was performed as described earlier (22). Briefly, a bacterial culture greater than $2x10^8$ diversity prepared by transformation with the plasmid DNA library was infected with VSC M13 helper phage (Stratagene) at 50 moi to generate a phage display library. The phage library was panned by affinity binding on DENV-1, DENV-2, or DENV-3 virions captured by chimpanzee dengue virus-convalescent sera coated on the wells of an ELISA plate. Following three cycles of panning, the selected phage was used for infection of *E. coli* XL-1 to produce phagemid DNA. Phagemid DNA was cleaved with SpeI and NheI to remove the phage gene III segment and circularized prior to use for transformation of *E coli* XL-1. *E. coli*

colonies were screened by ELISA to identify clones that yielded soluble Fab fragments reactive with DENV-1, DENV-2 or DENV-3.

DNA sequencing of dengue virus-specific Fab clones.

Plasmid from selected *E. coli* clones producing soluble Fabs was first analyzed by digestion with BstN1 to identify clones with distinct cleavage patterns. Sequence analysis of the V_H and V_L DNA inserts was performed on an automated DNA sequencer using a Taq fluorescent dideoxynucleotide terminator cycle sequencing kit. The following primers were used: 5' ACAGCTATCGCGATTGCAGTG and 5' CACCTGATCCTCAGATGGCGG for sequencing the V_L segments; 5' ATTGCCTACGGCAGCCGCTGG and 5' GGAAGTAGTCCTTGACCAGGC for sequencing the V_H segments. Software Vector NTi Suite 7.0 (InforMax) was used for analysis of the sequences. The DNAPLOT software program (MRC Center for Protein Engineering) was used for a homologous sequence search of the human IgG variable segments in the data bank.

Fab production and purification

Selected *E. coli* clones were grown in 2 liters of L-broth containing 1% glucose and 100 μg/ml ampicillin and 10 μg/ml tetracycline to an early log phase at 30 C. The bacteria were pelleted and resuspended in 2 liters of L-broth containing ampicillin and tetracycline plus 0.1 mM inducer isopropyl-β-D-thiogalactopyranoside (IPTG) for growth at 30 C for 4-5 hr (13, 35). After induction, the bacteria were collected and resuspended in 40 ml of buffer containing 50 mM phosphate buffer, pH 8.0, 10 mM Tris-HCl, 100 mM NaCl, and 0.1 mM protease inhibitor 4-(2-aminoethyl)-benzene sulfonyl fluoride (AEBSF). After three cycles of freezing and thawing to release the soluble Fab product from the bacterial periplasm, clear supernatant was prepared by centrifugation at 12,000 rpm in a Beckman JA-20 rotor for 60 min. The histidine-tagged Fab was affinity-purified through a column of TALON Metal Affinity Resin (Clontech). The purity of the Fab preparation was verified by polyacrylamide gel electrophoresis and the Fab concentration determined by ELISA using human IgG $F(ab')_2$ (Cappel) as a protein weight standard.

Biotinylation of purified Fab fragments and competition ELISA.

Purified Fab was biotinylated with EZ-Link NHS-LC-Biotin (Pierce) as suggested by the supplier. After extensive dialysis against PBS, the biotin-labeled Fab was analyzed for binding to DENV-1 or DENV-2 coated on wells of a microtiter plate. For competition ELISA, a fixed concentration of biotin-labeled Fab was mixed with the crude or purified preparation of competing Fabs in serial dilutions. The mixture was added to DENV-1 or DENV-2 coated wells and incubated at 37 C. After washing, streptavidin-alkaline phophatase (Pierce) was added for detection of biotinylated Fab reactive to DENV-1 or DENV-2 as described (23).

Western blot analysis.

Virus samples were prepared by mixing approximately 10^5 pfu of each virus with an equal volume of 2x sample buffer containing 2% SDS, 20% glycerol, 20mM Tris-HCl, pH 8.0 and 0,03 % bromophenol blue. The sample was loaded on SDS-polyacrylamide gel and separated by electrophoresis. The gel was blotted on a nitrocellulose membrane, treated with 5% skim milk, reacted with Fab 1A5 and then with a 1/1000 dilution of goat anti-human IgG-horseradish peroxides (Pierce). The blot was developed with Sigma fast 3,3'-diaminobenzidine (Sigma-Aldrich).

Measurement of neutralizing activity of chimpanzee Fab fragments.

Purified Fab antibodies were used in PRNT to determine the neutralizing titer against each of the four dengue virus serotypes. Typically, approximately 50 pfu of the dengue virus in 100 µl of MEM was mixed with the same volume of the Fab in serial dilution. The dengue virus-Fab antibody mixture was incubated at 37 C for 30 min and then added to confluent Vero cells in a 24-well plate. After a 30-min adsorption period at room temperature, an overlay of MEM containing 2% FCS and 1% Tragacanth gum was added and the plates were placed in a 5% CO_2- incubator at 37 C for 3-4 days. Virus foci that formed on the cell monolayer were immuno-stained (28). The $PRNT_{50}$ titer in µg/ml was calculated. Neutralization of the BSL-3 flaviviruses TBEV, JEV and WNV was performed with attenuated BSL-2 variants LGTV, the JEV strain SA 14-14-2 and the

WNV/DENV-4 chimera, which contains the WNV preM-E structure protein genes on the DENV-4 backbone.

Construction of recombinant plasmid and expression of whole IgG1 molecules in CHO cells.

The pFab CMV-*dhfr* vector for full-length IgG1 expression was constructed from plasmid pFab CMV originally obtained from P. Sanna (Scripps Research Institute) (34). A di-hydrofolate reductase gene (*dhfr*) along with the transcription signals was inserted at the unique Not I site as a selecting marker and for gene copy amplification. In addition, an A to G substitution at the last nucleotide position of the intron that preceded the C_H3 exon present in the original vector was made to enable full-length IgG1 expression (23). Fabs 2H7, 1A5, 3A2, and 1B2 were selected for conversion to whole IgG1 antibodies for analysis of their neutralizing activity. The V_L DNA segment of each Fab was inserted into the expression vector at the SacI and XbaI sites. The V_H DNA segment of the Fab, regenerated by PCR, was then added at the XhoI and SpeI sites. The chimpanzee-specific sequences in the hinge region were converted to the human sequence as described (23).

Production of whole IgG molecules in CHO/dhfr- cells (ATCC) was carried out by transfection with RsrII-linearized plasmid in the presence of Lipofectamine (Gibco). Two days after transfection, cells in a T25 flask were re-plated in Iscove's Modified Dulbecco Medium supplemented with 10% FBS plus 10^{-7} M methotrexate (MTX) in the absence of hypoxanthine/thymidine as selecting medium (7, 42). Colonies of CHO cells resistant to $2x10^{-7}$ M MTX appeared approximately two weeks after transfection. The transformed CHO cells secreting IgG1 in the medium were identified following cloning in a 96- or 24-well plate. To produce IgG1, the selected CHO cells were adapted to grow in CHO CD medium. The culture medium was concentrated and the IgG1 product was purified through a protein-A affinity column (Pierce). The apparent affinity constant (*Kd*) for the binding of the IgG to each of the four dengue virus serotypes was calculated as the antibody concentrations that gave 50% maximum binding by ELISA (21, 25).

Results

Chimpanzee γ1/κ antibody library and identification of Fabs recovered by panning with

DENV-1, DENV-2 or DENV-3.

As described earlier, two chimpanzees (# 1616 and # 1618) that had been intra-hepatically transfected with infectious DENV-4 RNA were infected with a mixture of DENV-1, DENV-2 and DENV-3 nine-and-half months later. Each of the chimpanzees developed moderate to high $PRNT_{50}$ titers of antibodies against DENV-1, DENV-2 and DENV-3. The $PRNT_{50}$ titer against DENV-4 also increased appreciably after secondary dengue infection. Chimpanzee 1618 developed slightly higher neutralizing antibody titers against DENV-1, DENV-2 and DENV-3 than did chimpanzee 1616 (23). Previously, we constructed a phage library from bone marrow mRNA of chimpanzee 1618 and identified DENV-4 and dengue-complex specific Fabs following panning of the library against DENV-4 (23). Based on this experience, we reasoned initially that separate panning of the phage library using DENV-1, DENV-2 or DENV-3 would yield dengue type, sub-complex or complex-specific Fab clones that could be further analyzed for their capacity to neutralize DENV-1, DENV-2 or DENV-3 *in vitro*.

(a) Fabs recovered from panning against DENV-1. Several Fab clones with distinct BstN1 digestion patterns were recovered following panning with DENV-1. PRNT against DENV-1 was carried out to identify the most promising neutralizing Fab antibodies. Fab clones that did not neutralize DENV-1 or only poorly neutralized it, were not studied further. Table 1 shows that Fab 2H7 and Fab 2H5 efficiently neutralized DENV-1 at a $PRNT_{50}$ titer of 0.26 and 0.47 μg/ml, respectively. Unexpectedly, each of these Fabs also neutralized DENV-2 at a titer similar to that detected for DENV-1. The $PRNT_{50}$ titer of these Fabs against DENV-3 or DENV-4 was reduced by 20 fold or more. Fab 2H5 and Fab 2H7 shared similar sequences (see below), but Fab 2H5 neutralized all four dengue viruses at lower titers than did Fab 2H7. Fab 2H5 was therefore not studied further.

(b) Fabs recovered from panning against DENV-2. Three distinct neutralizing Fabs, i.e., 1A5, 1A10, and 1B2, were identified in this experiment (Table 1). Like Fab 2H7 and Fab 2H5 identified above, Fab 1A5 efficiently neutralized both DENV-1 and DENV-2 at a similar $PRNT_{50}$ of 0.49 and 0.77 μg/ml, respectively, and also neutralized DENV-3 and DENV-4, but at a lower titer. Fab 1B2 and Fab 1A10 neutralized DENV-1 more efficiently than DENV-2 and much more efficiently than DENV-3 or DENV-4.

(c) Fabs recovered from panning against DENV-3. A large number of Fab clones

showing a distinct BstN1 digestion pattern were recovered from the library by panning against DENV-3. Fab 3A2 neutralized DENV-1 and DENV-2 at a titer of 0.37 and 1.33 μg/ml, respectively and also efficiently neutralized DENV-3 at a $PRNT_{50}$ titer of 3.0 μg/ml (Table 1). The ability of Fabs to cross-neutralize DENV-1 and DENV-2 at a similar high titer was a novel characteristic of several monoclonal antibodies, regardless of the dengue virus serotype used as the panning antigen.

Analysis of V_H and V_L sequences.

The amino acid sequences in the V_L and V_H segment of six selected Fab antibodies are shown in Figs. 1A and 1B. Fabs 2H7, 2H5, 1A5 and 3A2 were closely related, as an identical or nearly identical sequence was present in various framework segments or complementarity determining regions (CDR's) of the light chain or the heavy chain. Nevertheless, minor sequence variations (two or more amino acids) among them were present in other regions of the heavy chain as well as some regions of the light chain. These Fabs contained an identical or nearly identical 16-amino acid sequence, which included two cysteines in the CDR3-H domain principally involved in antigen binding. The sequences of Fab 1B2 and Fab 1A10 were distinct and contained a CDR3- H sequence different from those of Fabs 2H7, 1A5 and 3A2. Table 2 shows the result of a homologous sequence search of human IgG germ line gene segments most related to the V_H or V_L segments of the selected six chimpanzee Fabs. The germ line origin was the same for Fab 2H7, 2H5, 1A5, or 3A2 and the homology with the most related human sequence was 82-94%, excluding the CDR3-H and CDR3-L regions.

The V_H and V_L sequences of these Fab antibodies were also compared with the corresponding sequence of the Fab antibodies previously recovered by panning with DENV-4. Interestingly, Fab 1A10 and Fab 3E4 shared an identical V_H sequence with the exception of two amino acids: one in the FR1 region and the other in the CDR3 region (Fig. 1B). These two Fabs, however, differed appreciably in various regions of the V_L sequence (Fig. 1A). While the neutralizing activity of Fab 3E4 against DENV-1 and DENV-2 was low (titer greater than 42 μg/ml), Fab 1A10 neutralized DENV-1 and DENV-2 at a titer of 0.94 and 5.26 μg/ml, respectively.

Antigen specificity of chimpanzee Fabs.

Soluble Fabs were analyzed for binding activity to each of the four dengue virus serotypes by ELISA. Table 3 shows that each of these Fabs was broadly cross-reactive for all four dengue serotypes and had a similar high binding titer. Surprisingly, none of the Fab antibodies recovered from panning with DENV-1, DENV-2 or DENV-3 reacted in a dengue type-specific manner.

Radio-immunoprecipitation was performed to determine the antigen binding specificity for each of the Fabs using a radio-labeled lysate of Vero cells infected with DENV-1, DENV-2, DENV-3 or DENV-4. Fig. 2 shows a typical auto-radiogram of the immune precipitate separated by polyacrylamide gel electrophoresis. Fab 1A5 and Fab 1A10 specifically precipitated the E protein, migrating as a doublet, of each of the four dengue viruses. Fabs 2H7, 3A2 and 1B2 also precipitated E from the lysate of each of the four dengue virus serotypes (data not shown).

Analysis of chimpanzee Fabs binding to DENV-1 or DENV-2 by competition ELISA.

Fabs 1A5, 2H7 and 3A2 shared an identical or nearly identical CDR3-H sequence, whereas Fab 1A10 and Fab 1B2 each contained a distinct CDR3-H sequence. The relatedness of the binding sites for Fabs 1A5, 1A10 and 1B2 on DENV-1 or DENV-2 was analyzed by competition ELISA. Surprising, binding of affinity-purified, biotinylated Fab 1A10 to DENV-1 was competed by the unlabeled crude preparation of Fab 1B2 and Fab 1A5 (Fig. 3A). Similarly, binding of biotinylated Fab 1A5 to DENV-1 was competed by Fab 1B2 and Fab 1A10 and binding of Fab 1B2 was competed by Fab 1A5 and Fab 1A10 (Fig. 3B and 3C). When DENV-2 was tested, the binding competition patterns among these three Fabs were essentially identical to that seen with DENV-1 (Fig. 3D, 3E, and 3F). As a control, chimpanzee Fab 1F2, which did not bind either DENV-1 or DENV-2, failed to compete any of the labeled Fabs. Thus, the site that was occupied by Fab 1A5 overlapped with the site occupied by Fab 1B2 and Fab 1A10 on DENV-1, and on DENV-2 E. The Fab 1A5 binding site (epitope) on the DENV-2 E protein was mapped in a separate study (14).

Cross-reactivity of chimpanzee Fabs to WNV and other flaviviruses.

In the course of this study, we found that the Fabs recovered in this study also reacted with the WNV/DENV-4 chimera at a high titer as detected by ELISA (data not shown). Fab 1A5 was selected for analysis of binding to the four dengue viruses and other major flaviviruses. Western blot analysis (Fig. 4) showed that Fab 1A5 reacted relatively strongly with each of the four dengue viruses and WNV/DENV-4. By comparison, Fab 1A5 bound weakly to JEV SA14-14-2 and LGTV TP 21. The reduced binding activity of Fab1A5 to the JEV SA14-14-2 and LGTV TP21 reflected the low $PRNT_{50}$ titer (greater than 70 μg/ml) of Fab 1A5 against these two viruses. Interestingly, Fab 1A5 neutralized WNV/DENV-4 chimera at a $PRNT_{50}$ titer of 4.8 μg/ml, similar to that measured for DENV-3 and DENV-4.

Production and characterization of full-length humanized IgG1 antibodies

With the exception of Fab 1A10, the Fab fragments were each converted to the full-length IgG1 antibody in combination with the human IgG1 sequence using the expression vector pFab CMV-*dhfr* for transformation of CHO cells (23). Among these antibodies, IgG1 1A5 was produced in the highest yield, approximately 2 μg/10^6 cells per day in the medium of the transformed CHO cells. IgG1 1A5 was selected to determine the $PRNT_{50}$ against each of the four dengue viruses (Fig. 5). IgG1 1A5 neutralized DENV-1 and DENV-2 at a $PRNT_{50}$ titer of 0.48 and 0.95 μg/ml, respectively. IgG1 1A5 also neutralized DENV-3 and DENV-4 at a $PRNT_{50}$ titer of 3.2 and 4.3 μg/ml, respectively. The apparent affinity constants, *Kd*, were calculated at 0.50, 0.60, 0.67 and 0.82 nM for DENV-1, DENV-2, DENV-3 and DENV-4, respectively, in the same decreasing order as the $PRNT_{50}$ titers against these viruses. Humanized IgG1 1A5 was also tested for neutralization of WNV/DENV-4, JEV strain SA14-14-2 and LGT strainTP21 by PRNT. The $PRNT_{50}$ titer against WNV/DENV-4 was 3.8 μg/ml, whereas the $PRNT_{50}$ titer against JEV strain SA14-14-2 and LGTV strain TP21 was 21 and 28 μg/ml, respectively (Fig. 5).

Discussion

A safe and effective vaccine for prevention of dengue virus infection is still not available. Passive immunization with neutralizing antibodies represents an attractive

alternative to a dengue vaccine. Repertoire cloning of bone marrow mRNA from chimpanzees infected with all four dengue virus serotypes has been performed to identify Fab antibody fragments that could be used for development of clinically acceptable neutralizing antibodies. In our earlier study, serotype-specific Fab antibodies that efficiently neutralized DENV-4 were identified using DENV-4 as panning antigen. A full-length humanized antibody, designated as IgG1 5H2, derived from one of these Fabs, has proven to be highly potent for neutralization of DENV-4 (23). This success prompted us to use the same library for recovery and identification of neutralizing Fab antibodies against each of the other three dengue virus serotypes. A large panel of Fabs was recovered by panning of the phage library with DENV-1, DENV-2 or DENV-3. Regardless of the panning antigen used, recovered Fabs were shown to be cross-reactive to all four dengue serotypes and serotype-specific neutralizing Fabs against DENV-1, DENV-2 or DENV-3 were not identified. Several Fab antibodies, as exemplified by Fabs 1A5, 2H7 and 3A2, were highly efficient for cross-neutralization against DENV-1 and DENV-2. Each of these Fab antibodies also neutralized DENV-3 and DENV-4, but at a reduced titer. These chimpanzee Fabs appear to represent a novel class of dengue complex cross-neutralizing monoclonal antibodies that have not been described before for mouse monoclonal antibodies.

It is reasonable to assume that the responses of the chimpanzees to primary infection with DENV-4 and to secondary dengue infection with a mixture of DENV-1, DENV-2 and DENV-3 are represented in the respective genetic repertoire of the phage libraries. A number of dengue cross-reactive Fab antibodies were identified from chimpanzee 1618 following primary infection, although their neutralizing activities were low or not detected (23). The secondary infection may have selected for the production of cross-reactive antibodies in the chimpanzee because of memory T cells and B cells. Evidence for such immune responses is provided by comparison of the sequences between Fab 3E4, recovered from primary infection with DENV-4, and Fab 1A10 recovered from the same chimpanzee after secondary infection with DENV-1, DENV-2 and DENV-3. Fab 3E4 and Fab 1A10 shared a nearly identical V_H sequence, although their V_L sequences varied. Fab 3E4 was cross-reactive to all four serotypes and was poorly neutralizing against each of these viruses (23). In contrast, Fab 1A10 neutralized DENV-1 and

DENV-2 at a titer much higher than that measured for Fab 3E4 (Table 1). This situation is reminiscent of the antigenic response termed "original antigenic sin", first described for influenza infections (41).

The sequence similarity among Fabs 1A5, 2H7, 2H5 and 3A2 reflects their neutralizing activities against each of the four dengue serotype viruses. Characteristically, each of these Fabs contains a 16-amino acid sequence in CDR3-H, which is longer than the sequence of 8-14 residues found in most human CDR3-H or the sequence of 8-12 residues in mouse CDR3-H sequences (43). Also, there are two Cys residues separated by four amino acids in the chimpanzee CDR3-H sequences. It is speculated that the two additional Cys could form a local disulfide bond, as all other Cys residues in Fab fragments or full-length antibodies participate in disulfide bond formation. Formation of this additional disulfide bridge could impose an additional constraint on the flexibility of the CDR3-H loop.

Despite their sequence differences, Fab 1A10, Fab 1B2 and Fab 1A5 competed with each other for binding to DENV-1 and DENV-2 in competition ELISA. This suggests that the binding sites of these apparently different Fabs on these viruses are spatially close or overlapping. It is also possible that the binding site on the surface of DENV-1 and DENV-2 E for each of these Fabs is unique, but binding of one Fab results in alteration of the binding sites for others through a steric hindrance. Interestingly, Fab 1A10 and Fab 1B2 neutralized DENV-1 more effectively than DENV-2 even though they were derived by panning against DENV-2.

Sequence analysis indicates that there is a high degree of similarity between the chimpanzee V_H and V_L sequences and their human germline homologs (82% or greater). The sequence homology in the constant regions C_H1 and C_L between human and chimpanzee is even greater, approaching 100% (8, 10). The high level of antibody sequence similarity suggests the possibility that the humanized chimpanzee antibodies may be administered directly to humans without further modifications. Experimental data available indicate that little immunogenicity is seen when components of human antibodies are introduced into rhesus monkeys or chimpanzees (9, 27).

We have reported that the full-length humanized antibody IgG1 5H2 has a DENV-4 neutralizing $PRNT_{50}$ titer of 0.03-0.05 μg/ml, compared to the titer of 0.24-0.52 μg/ml

measured for Fab 5H2 (23). On a molar basis, the neutralizing activity of IgG1 5H2 is approximately 30-40 fold higher than the Fab 5H2 fragment. A similar increase of neutralizing activity for mouse monoclonal antibody 4E11, compared to its derived Fab fragment against DENV-1, has been reported (37). A comparison of the neutralizing activities for several influenza virus HA monoclonal antibodies and their Fab fragments derived by papain cleavage has also been reported (36). This study has shown that most IgGs have a greater than 90 fold increase of neutralizing activities compared to their Fabs. Based on these data, we had hoped that the full-length IgG1 1A5 antibody would have a neutralizing titer 30-40 fold higher than the Fab 1A5 fragment against DENV-1 or DENV-2. This turned out not to be the case, as there was only a 3 fold increase of neutralizing activity for IgG 1A5 over Fab 1A5 against each of the four dengue virus serotypes on a molar basis. A similar level of increase of activity has also been reported for the full-length IgG1 converted from a neutralizing Fab against the Ebola virus (22). The relative neutralizing activity between the monovalent Fab and bivalent IgG is probably dependent on accessibility to the epitope of the virus it recognizes.

The cause of severe dengue, which is sometimes associated with secondary infection and sometimes with primary infection, remains controversial. According to one hypothesis, the increased dengue severity is an immunopathological phenomenon caused by antibody-dependent enhancement of infection. In a secondary infection dengue virus could complex with a sub-neutralizing concentration of cross-reactive antibodies produced during the primary infection, leading to an increased uptake and replication in susceptible mononuclear cells via their Fc receptors. This issue is particularly important for dengue prophylaxis with antibodies such as IgG1 1A5 in view of their cross-reactivity to all four dengue serotypes. Several FcRγ receptors have been identified on the cell surface and their binding sites on the respective IgG have been identified (1, 4). It may be possible to diminish the binding affinity between the Fc portion of the antibody and cellular receptors by altering the FcRγ1 binding sequences in the IgG1 1A5.

Antibody IgG1 1A5 could be an attractive candidate for the development of a passive immunization strategy against dengue. One of the attractions is the use of IgG1 1A5 for cross-neutralization of DENV-1 and DENV-2. Administration with a dose of 2 mg/kg of the antibody would give a serum titer of approximately 40 and 20 $PRNT_{50}$ against

DENV-1 and DENV-2, respectively. In addition, IgG1 1A5 also neutralized DENV-3, DENV-4 and WNV at a similar $PRNT_{50}$ titer, ranging from 3.2 to 4.2 μg/ml. WNV is becoming increasingly important for the public health of the US and many other countries. It has been shown in a mouse model that passive administration of immune mouse sera or human immune gamma globulin protected against lethal WNV challenge (11). Administration of human gamma globulin also improved the clinical outcome after the virus had disseminated in the CNS in mice. Humanized IgG1 1A5 may therefore prove to be a valuable candidate not only for prophylactic but also for therapeutic application against this virus.

It should be pointed out that the neutralizing activity of Fab 1A5 and IgG1 1A5 against JEV strain SA14-14-2 and LGTV strain TP21 was lower than that against WNV/DENV-4. The neutralizing titers of IgG 1A5 against the wild type JEV and other members of the TBEV remain to be determined. The amino acid sequence of the attenuated JEV strain SA14-14-2 differs from that of its parental virulent virus in a number of positions, including several in E (26). Mapping of the epitope determinants of Fab 1A5 on the DENV-2 E is presented in the accompanying paper (14). The results of that study provide evidence for an explanation that the reduced neutralizing activities of Fab 1A5 against these viruses stems from the sequence variations at or near the mapped determinant.

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Figure legends

Fig. 1. Amino acid sequences of Fabs. (A) sequences of the V_L κ light chain segments; (B) sequences of the VH γ1 heavy chain segments. FR, framework region; CDR, complementarity-determining region. The dash symbol represents an amino acid deletion and an identical amino acid is indicated by a dot. The sequence of Fab 3E4 described previously (23) was included for comparison with that of Fab 1A10.

Fig. 2. Analysis of antigen specificity by radio-immunoprecipitation. Radioactive ^{35}S-methionine-labeled lysates separately prepared from Vero cells infected with each of the dengue virus serotypes (D1 to D4) were used for immune precipitation with Fab 1A5 or Fab 1A10. M shows the protein markers with molecular weight in kD on the left. Each of the Fabs precipitated the E protein of each of four dengue virus serotypes. Note that the E protein often migrated as a doublet or a broad band probably resulting from differences in glycosylation.

Fig. 3. Analysis of Fab binding to DENV-1 or DENV-2. Fabs 1A5, 1B2 and 1A10 were affinity-purified, biotinylated and used for analysis of binding activity to DENV-1 or DENV-2 virus by competition ELISA in the presence of competing, unlabeled Fabs. Chimpanzee Fab 1F2, which did not react with any of the dengue viruses, was used as a negative control. The numbers on the Y axis are OD readings and the X coordinates represent reciprocal dilutions of the competing Fabs. D1 or D2 (top of each panel) indicates DENV-1 or DENV-2 used. The insert inside panel A shows the symbol for each Fab and the symbols are the same for all six panels.

Fig. 4. Binding of Fab 1A5 to dengue viruses and other flaviviruses as measured by Western blotting. Approximately 10^5 pfu of each virus was applied and separated by polyacrylamide gel electrophoresis. Gel lanes are: D1, DENV-1; D2, DENV-2; D3, DENV-3; D4, DENV-4; WN/D4, WNV/DENV-4 chimera; JE, JEV strain SA 14-14-2; LGT, LGTV strain TP 21. The position of the E protein is indicated. Molecular size markers are shown on the left.

Fig. 5. In vitro neutralization of dengue viruses and other flaviviruses by humanized IgG1 1A5. The neutralizing activity of IgG1 1A5 against DENV-1, Hawaii strain; DENV-2, New Guinea B strain; DENV-3, H87 strain; DENV-4, strain 814669; JEV, vaccine strain SA14-14-2; LGTV, strain TP 21; WNV/DENV-4 chimera was analyzed by PRNT.

Table 1. Dengue virus cross-neutralizing activities of Fabs identified by panning against DENV-1, DENV-2, or DENV-3

Fab	Panning antigen	$PRNT_{50}$ titer (μg/ml) against			
		DENV-1	DENV-2	DENV-3	DENV-4
2H7	DENV-1	0.26	0.33	5.92	7.26
2H5	DENV-1	0.47	0.53	20.8	9.26
1A5	DENV-2	0.49	0.77	3.49	4.23
1A10	DENV-2	0.94	5.26	26.3	12.6
1B2	DENV-2	0.50	3.13	> 100	29.2
3A2	DENV-3	0.37	1.33	2.99	4.71
3E4*	DENV-4	42.7	>100	>100	40.5

Fabs which are underlined shared a similar V_H sequence or V_L sequence.

* Fab 3E4 was recovered from the chimpanzee after primary infection by panning against DENV-4 as described previously (23). Fab 3E4 was included for comparison with Fab 1A10.

Table 2. Sequence similarity between chimpanzee Fab antibodies and their most related human immunoglobulin homologs

Chimp. Fab	V_H vs Human homolog Family (gene)	% identity	Ref. cited	V_L vs Human homolog Family (gene)	% Identity	Ref. cited
2H7	VH3 (8-1B)	84	3	VKI (DPK9)	90	6
2H5	VH3 (8-1B)	85	3	VKI (DPK9)	86	6
3A2	VH3 (8-1B)	82	3	VKI (DPK9)	86	6
1A5	VH3 (8-1B)	85	3	VKI (DPK9)	86	6
1B2	VH4 (DP-78)	87	5	VKI (Va)	94	30
1A10	VH1 (DP-10)	86	38	VKII (A2b)	85	12

The DNAPLOT program was used to search for the most homologous sequence of human germ-line IgG genes in the data base. Percent amino acid identity in the V_H or V_L segment excluding the CDR-3 region is indicated.

Table 3. Binding activities of Fab monoclonal antibodies to each of the four dengue virus serotypes as determined by ELISA

	ELISA titer of Fab binding to			
Fab	DENV-1	DENV-2	DENV-3	DENV-4
1A5	4.1	3.8	3.8	3.8
3A2	4.1	3.8	3.8	3.8
2H7	4.1	3.8	3.6	3.8
1B2	3.9	3.8	3.8	3.8
1A10	4.1	3.8	3.6	3.8
5H2	<1.0	<1.0	<1.0	3.8
3E4	4.0	3.8	3.6	3.8
1F2*	<1.0	<1.0	<1.0	<1.0

Microtiter plates were coated with DENV-1, DENV-2, DENV-3 or DENV-4 virions. Data are presented as $\log_{10}$ of the reciprocal dilution that gave an OD reading 2 fold or higher than the background. Dengue virus cross-reactive Fab 3E4 and DENV-4 specific Fab 5H2 were described previously (23). The starting concentration of each Fab was approximately 140 μg/ml.

* Chimpanzee Fab 1F2 was used as negative control.

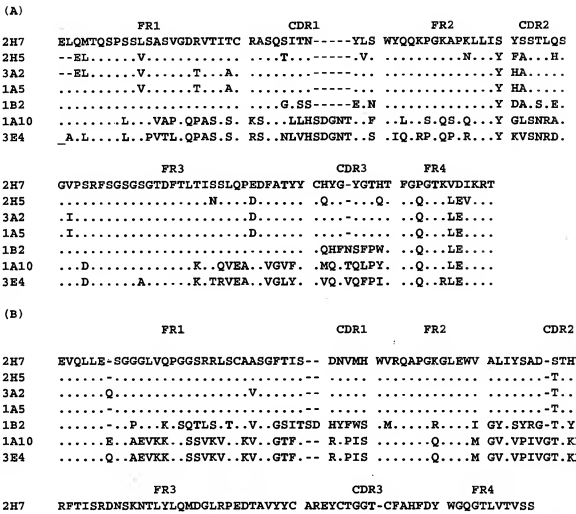
(A)
FR1 CDR1 FR2 CDR2
2H7 ELQMTQSPSSLSASVGDRVTITC RASQSITN-----YLS WYQQKPGKAPKLLIS YSSTLQS
2H5 --EL.....V..... ....T...-----.V. .....N...Y FA...H.
3A2 --EL.....V.....T...A. .....-----... .....Y HA.....
1A5 .....V.....T...A. .....-----... .....Y HA.....
1B2 ....G.SS-----E.N .....Y DA.S.E.
1A10 .....L...VAP.QPAS.S. KS...LLHSDGNT..F ..L..S.QS.Q...Y GLSNRA.
3E4 _A.L....L..PVTL.QPAS.S. RS..NLVHSDGNT..S .IQ.RP.QP.R...Y KVSNRD.
FR3 CDR3 FR4
2H7 GVPSRFSGSGSGTDFTLTISSLQPEDFATYY CHYG-YGTHT FGPGTKVDIKRT
2H5 .....N....D..... .Q..-...Q. ..Q...LEV...
3A2 .I.....D..... ....-..... ..Q...LE....
1A5 .I.....D..... ....-..... ..Q...LE....
1B2 ..... .QHFNSFPW. ..Q...LE....
1A10 ...D.....K..QVEA..VGVF. .MQ.TQLPY. ..Q...LE....
3E4 ...D.....A.....K.TRVEA..VGLY. .VQ.VQFPI. ..Q..RLE....
(B)
FR1 CDR1 FR2 CDR2
2H7 EVQLLE-SGGGLVQPGGSRRLSCAASGFTIS-- DNVMH WVRQAPGKGLEWV ALIYSAD-STHYADSVKG
2H5 .....-.....-- ....-T.....
3A2 .....Q.....V.....-- ....-T.....
1A5 .....-.....-- ....-T.....
1B2 .....-..P...K.SQTLS.T..V..GSITSD HYFWS .M.....R....I GY.SYRG-T.Y.NP.L.S
1A10 .....E..AEVKK..SSVKV..KV..GTF.-- R.PIS .....Q....M GV.VPIVGT.KH.QKFQ.
3E4 .....Q..AEVKK..SSVKV..KV..GTF.-- R.PIS .....Q....M GV.VPIVGT.KH.QKFQ.
FR3 CDR3 FR4
2H7 RFTISRDNSKNTLYLQMDGLRPEDTAVYYC AREYCTGGT-CFAHFDY WGQGTLVTVSS
2H5 .....S..... .....D.-..... .....S...
3A2 .....S..... .....G.-..... .....T...
1A5 .....S..... .....D.-..... .....S...
1B2 .V.M.VTAA..... ..ASV.AGMPAAGTL.H .....
1A10 RV.IIA.ESTSTAYMELSS..S..... .TYYAD.----SSYSEY .....
3E4 RV.IIA.ESTSTAYMELSS..S..... .TYYADV----SSYSEY .....

Fig.1

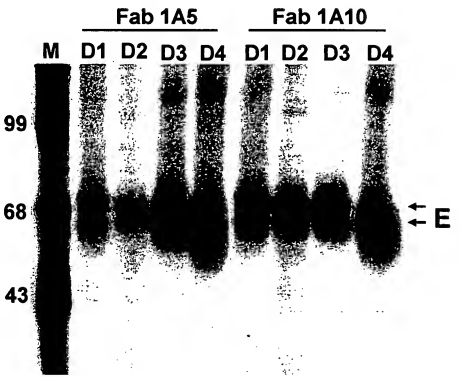

Fig. 2.

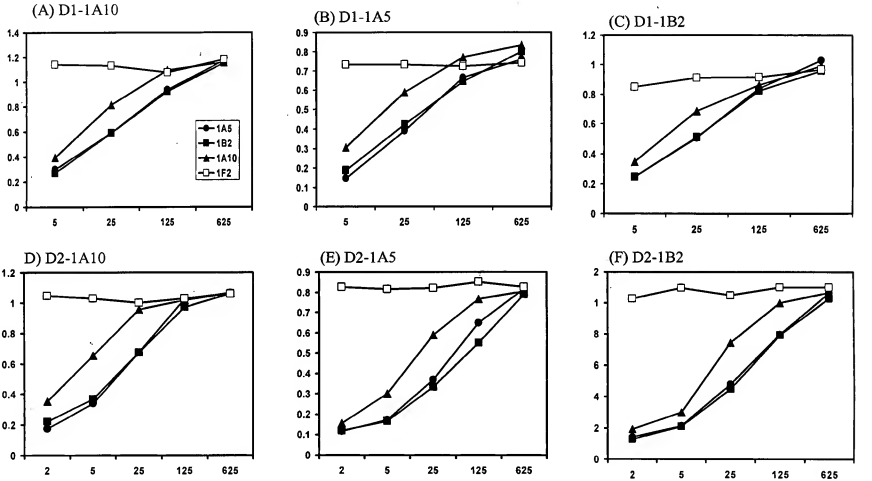
(A) D1-1A10
1A5
1B2
1A10
1F2
(B) D1-1A5
(C) D1-1B2
D) D2-1A10
(E) D2-1A5
(F) D2-1B2

Fig. 3.

Fig. 4

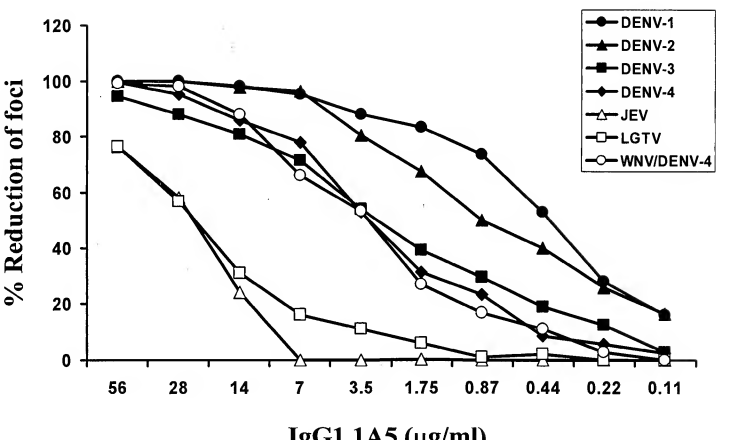
% Reduction of foci
120
100
80
60
40
20
0
56
28
14
7
3.5
1.75
0.87
0.44
0.22
0.11
IgG1 1A5 (μg/ml)
DENV-1
DENV-2
DENV-3
DENV-4
JEV
LGTV
WNV/DENV-4

Fig. 5

Epitope Determinants of a Chimpanzee Fab Antibody that Efficiently Cross-Neutralizes Dengue Type 1 and Type 2 Viruses Map to Inside and in Close Proximity to the Fusion Loop of the Dengue Type 2 Virus Envelope Glycoprotein

Summary

The epitope determinants of chimpanzee Fab antibody 1A5, which had been shown to be broadly reactive to flaviviruses and efficient for cross-neutralization of dengue type 1 and type 2 viruses (DENV-1 and DENV-2), were studied by analysis of DENV-2 antigenic variants. Sequence analysis showed that one antigenic variant contained a Gly-to-Val substitution at position 106 within the flavivirus-conserved fusion peptide loop of the envelope protein (E) and another variant contained a His-to-Gln substitution at position 317 in E. Substitution of $Gly_{106}Val$ in DENV-2 E reduced the binding affinity of Fab 1A5 by approximately 80 fold, whereas substitution of $His_{317}Gln$ had little or no effect on antibody binding as compared to the parental virus. Treatment of DENV-2 with β-mercaptoethanol abolished binding of Fab 1A5, indicating that disulfide bridges were required for the structural integrity of the Fab 1A5 epitope. Binding of Fab 1A5 to DENV-2 was competed by an oligopeptide containing the fusion peptide sequence as shown by competition ELISA. Both DENV-2 antigenic variants were shown to be attenuated or at least similar to the parental virus, when evaluated for growth in cultured cells or for neurovirulence in mice. Fab 1A5 inhibited low pH-induced membrane fusion of mosquito C6/36 cells infected with DENV-1 or DENV-2, as detected by reduced syncytium formation. Both substitutions in DENV-2 E lowered the pH threshold for membrane fusion, as measured by fusion-from-within assay. In the 3-D structure of E, Gly_{106} in domain II and His_{317} in domain III of the opposite E monomer were spatially close. From the locations of these amino acids, Fab 1A5 appears to recognize a novel epitope that has not been mapped before with a flavivirus monoclonal antibody.

Introduction

The four dengue virus serotypes (DENV-1 to DENV-4) constitute the dengue virus complex within the *Flavivirus* genus of the *Flaviviridae*. Dengue out-breaks and epidemics continue to pose a public health problem in most tropical and subtropical countries. Dengue illnesses range from mild dengue fever to severe dengue, characterized by dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), which has a high mortality rate. According to one estimate, approximately 50-100 million dengue infections and up to 250,000 DHF cases occur every year world-wide (11, 33). Despite six decades of research, a safe and effective dengue vaccine has not been developed, nor is a specific, short-term preventive measure available. Currently, prevention of dengue is carried out by mosquito vector control, which is rather ineffective. Several other arthropod-borne flaviviruses are also important human pathogens, including the yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), Japanese encephalitis virus (JEV) and West Nile virus (WNV), which has recently emerged in North America (22, 24). Vaccines against all of these viruses except WNV have been developed.

The flavivirus genome contains a positive strand RNA with one open reading frame coding for a polyprotein. The polyprotein is processed to produce the three structural proteins, i.e., the capsid (C), precursor membrane (prM) and envelope (E) proteins, plus seven nonstructural proteins, designated as NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The E protein is responsible for viral assembly and subsequent exit from the infected cells, viral attachment to the putative cell surface receptor(s), fusion with the endosomal membranes upon entry, and mediating protective immune responses in the infected host. Mouse monoclonal antibodies against the E proteins of most major flaviviruses have been identified (18, 41). Studies using these monoclonal antibodies have allowed identification of flavivirus group-, complex- and type-specific epitopes on the flavivirus E proteins. With few exceptions, neutralizing monoclonal antibodies are flavivirus type- or subtype-specific, consistent with the flavivirus classification determined with the polyclonal sera (7).

The 3-D structure of the flat homodimeric E glycoprotein that is organized in a direction parallel to the viral membrane has been determined for TBEV (39) and DENV-

2 (31). The E subunit, approximately 500 amino acids in length, is folded into three structurally distinct domains, termed domains I, II and III. Domain I organizes the entire E structure and contains a flavivirus-conserved glycosylated asparagine. Domain II is folded into an elongated structure containing at its distal end the fusion peptide sequence, commonly called the fusion loop, which is conserved among the flaviviruses. The outward glycan unit in domain I protrudes to cover the fusion loop of the other subunit. There is an extensive interface contact between domain II and each of the three domains of the neighboring subunit. Domain III is an immunoglobulin-like region and lies at the end of the subunit. The dimeric E structure realigns to become trimeric when triggered by lowering the pH, while the three domains remain intact structurally (6, 32). During the transition, the fusion loop becomes exposed and re-oriented outward, making it available for membrane contact.

Antigenic determinants of flavivirus cross-reactive antibodies have been mapped to domain II, whereas determinants of subtype- and type-specific antibodies have been assigned to domains I and III (18, 26, 41, 42). Most epitopes of neutralizing antibodies have been placed on the outer surface of the E glycoprotein, consistent with their accessibility to antibody binding. Mutations present in variant viruses that have escaped neutralization by antibodies blocking virus adsorption to Vero cells have been assigned to the lateral side of E in domain III (9). Similarly, the mutations of antigenic variants that affect mouse nuerovirulence have been mapped to this domain (8, 20, 21). These findings have suggested that the sequence in domain III may mediate viral attachment to the receptor on susceptible cells.

The antigenic model of flavivirus E proteins established thus far from studies with the large repertoire of mouse monoclonal antibodies has provided much information about serological specificities and functional activities (18, 41). The question remains whether these antigenic epitopes are mouse-specific or in fact, they represent immuno-dominant sites on E recognized by the immune systems of other host species as well. Unfortunately, there is a lack of flavivirus monoclonal antibodies from other host species, especially higher primates or humans.

We have recently turned to the identification of chimpanzee Fab fragments by repertoire cloning and construction of full-length humanized IgG antibodies in an effort

to develop a passive immunization strategy for prevention of dengue virus infection. We have described a DENV-4 specific chimpanzee Fab fragment and a derived full-length humanized IgG antibody highly efficient for neutralization of DENV-4 (30). We have also identified chimpanzee Fab fragments, including 1A5, that exhibited a broad cross-reactivity to members of the flavivirus group and cross-neutralized DENV-1 and DENV-2 efficiently (10). The current study describes mapping the epitope determinants of Fab 1A5 by analysis of DENV-2 antigenic variants. A determinant critically involved in Fab 1A5 antibody binding and neutralization mapped to Gly_{106} within the flavivirus-conserved fusion loop in domain II of E. Another determinant affecting antibody neutralization, but not antibody binding, mapped to His_{317} in domain III of the neighboring E monomer. Amino acid substitutions in these DENV-2 variants lowered the pH threshold for membrane fusion of the infected cells. From the locations of these amino acids in the 3-D structure, the Fab 1A5 antibody appears to recognize a novel epitope on E that has not been mapped before.

Materials and Methods

Dengue viruses and cultured cells. Simian Vero cells and mosquito C6/36 cells were grown in minimum essential medium (MEM) plus 10% fetal bovine serum (FBS), 2 mM L-glutamine, 0.05 mg/ml gentamycin, and 2.5 units/ml fungizone. Mouse-adapted DENV-2 New Guinea B (NGB) and New Guinea C (NGC) strains were used for selection of antigenic variants. DENV-2 NGC was provided by K. Eckels (5) and DENV-2 NGB at mouse passage 11 was provided by W. Schlesinger. Stocks of the dengue viruses were prepared from infected C6/36 cells grown in VP-SFM medium (Invitrogen). The titers of these viruses were approximately 1×10^7 plaque-forming units (pfu)/ml, determined on Vero cell monolayers.

Antibodies. Chimpanzee Fab 1A5 was identified by panning of a phage library using DENV-2 as described (10). Poly-histidine tagged Fab 1A5, expressed in *E. coli*, was affinity-purified using TALON affinity resin (Clontech). The concentration of Fab was determined colorimetrically using the BCA protein assay kit (Pierce). Hyper-immune mouse ascites fluid (HMAF) raised against DENV-2 and DENV-4 was purchased from

American Type Culture Collection. Mouse monoclonal antibody Mab 3H5, specific to DENV-2, was kindly provided by R. Putnak (19).

Plaque reduction neutralization test (PRNT). Approximately 50 pfu of DENV-2, or other viruses to be tested, were mixed with Fab 1A5 serially diluted in 250 µl of MEM. The mixture was incubated at 37 C for 1 h prior to use for infection of Vero cells or C6/36 cells in duplicate wells of a 24-well plate. Infected Vero cells were added with a medium overlay containing 1% gum tragacanth (Sigma) and incubated at 37 C for 3 days. Infected C6/36 cells were overlaid with medium containing 0.8% methyl cellulose and incubated at 32 C for 5 days. Foci of infected cells were visualized by immuno-staining, using HMAF and anti-mouse IgG peroxidase (Pierce). The Fab titer in µg/ml that produced 50% reduction of foci ($PRNT_{50}$) was calculated from at least 3 experiments.

Selection of DENV-2 antigenic variants. Affinity-purified Fab 1A5 was used for selection of antigenic variants from mouse-passaged DENV-2 NGB and DENV-2 NGC, both of which had been previously sequenced in the C-prM-E region (5; I. Tokimatsu, unpublished observations). Parental DENV-2 NGB or DENV-2 NGC, approximately $1x10^7$ pfu, was mixed with Fab 1A5 at 25 µg/ml (equivalent to 100 $PRNT_{50}$ titers) in MEM and incubated at 37 C for 1 h. The mixture was added to the Vero cell monolayer in a 35-mm culture plate for adsorption at 37 C for 1 h. The monolayer was rinsed once with phosphate buffered saline (PBS), 3 ml of MEM containing 2% FBS plus 5 µg/ml of Fab 1A5 was added and then the cells were incubated at 37 C for 7 days. Progeny virus in the culture medium was collected for neutralization with Fab 1A5, followed by infection of Vero cells again. The neutralization cycle was repeated and the Fab 1A5-resistance phenotype of progeny virus monitored. Fab 1A5-resistant variants were isolated by plaque-to-plaque purification three times on Vero cells prior to amplification in C6/36 cells.

Sequence analysis of antigenic variants. Genomic RNA of each antigenic variant was extracted using Trizol solution (Life Technologies). Reverse transcription of RNA with primer AGTCTTGTTACTGAGCGGATTCC at nucleotide positions 2587 to 2565 in

DENV-2 NS1 was carried out using the Superscript kit (Life Technologies). Amplification of C-prM-E DNA with appropriate primers by PCR was performed, using AmpliTaq DNA polymerase (Perkin-Elmer). The DNA product was sequenced using primers spanning the DNA segment in an ABI sequencer (Perkin-Elmer, Applied Biosystems). The sequences of eight to ten plaque-purified isolates from each variant were analyzed. Sequence assembly was performed using Vector NTI Suite (InforMax). Structural modeling of the mutant E protein was performed using SwissModel and the crystal coordinates of DENV-2 (1OAN.pdb) as the template (12, 31). Swiss-Pdb Viewer was used for graphical development.

Construction of DENV-2/DENV-4 chimeras. Construction of chimeric cDNA containing the C-prM-E sequence of parental DENV-2 NGB, DENV-2 NGC or their antigenic variants on the DENV-4 background was as described (4). Briefly, parental or variant DENV-2 C-prM-E DNA was generated by reverse transcription of virion RNA and PCR amplification. The DNA product was digested with BglII and XhoI and then cloned into plasmid p5'-2, replacing the corresponding DENV-4 sequence. The ClaI-XhoI fragment of p5'-2 DNA containing the DENV-2 C-prM-E sequence was then used to replace the corresponding fragment of full-length DENV-4 DNA, generating full-length chimeric DENV-2/DENV-4 DNA. Confluent C6/36 cells were transfected with the RNA transcripts of the chimeric DENV-2/DENV-4 DNA construct as described (4, 23). Three weeks after transfection, the culture medium had a titer greater than 10^6 pfu/ml determined by focus assay on C6/36 cells. The C-prM-E DNA segment of progeny virus was prepared for sequence verification.

Construction of DENV-4 variants. Two silent mutations, A to C at nucleotide 378 and C to T at nucleotide 381 near the fusion loop encoding sequence in E, were first introduced to create a unique AgeI site in full-length DENV-4 DNA (23). Site-directed mutagenesis by PCR was performed using a forward primer corresponding to nucleotides 8-33 of pBR322 and a reverse primer containing the AgeI cleavage sequence and following nucleotide substitution(s) in E: G to C at nucleotide 310 and G to A at nucleotide 311 for generating Gly_{104}His substitution; G to T at nucleotide 317 for Gly_{106}Val substitution;

and G to C at nucleotide 321 for Leu_{107}Phe substitution. The PCR products, digested with ClaI and AgeI, were each cloned into full-length DENV-4 DNA. RNA transcription and transfection of C6/36 cells and recovery of virus were performed as described above.

Polyacrylamide gel electrophoresis and Western blotting. Dengue virus was mixed with an equal volume of 2x sample buffer (2% SDS, 20% glycerol, 20 mM Tris-HCl, 0.02 % bromophenol blue) with or without 0.5% β-mercaptoethanol. When β-mercaptoethanol was used, the virus mixture was boiled for 10 min prior to loading for separation by polyacrylamide gel electrophoresis. Otherwise, the sample mixture was loaded directly. The protein gel was blot-transferred onto a nitrocellulose membrane electrophoretically. The protein blot was treated with 5% skim milk and reacted with Fab 1A5 or Mab 3H5 for 1 h. The blot was then washed with Tris-buffered saline containing 0.05% Tween 20 three times and reacted with goat anti-human IgG or anti-mouse IgG peroxidase (Pierce) at room temperature for 1 h. The protein blot was developed with Sigma fast™ 3,3'-diaminobenzidine (Sigma-Aldrich).

Antibody binding affinity assay. ELISA was performed to determine the binding affinity of Fab 1A5 to parental DENV-2 and its antigenic variants (25, 34, 37). Briefly, Mab 3H5-coated wells of a microtiter plate were blocked with 3% bovine serum albumin and then each virus was added to separate wells. Following incubation at 37 C for 1 h, affinity-purified Fab 1A5 in serial dilution was added and the plate incubated at 37 C for 1 h. Fab 1A5 bound to DENV-2 on the microtiter plate was detected using goat anti-human IgG alkaline phosphatase (Sigma). The apparent affinity constant *Kd* was calculated for the Fab 1A5 concentration in nM that produced 50% of maximum binding.

Binding of Fab 1A5 to oligopeptides. Three oligopeptides were analyzed: control peptide 1, GAMHSALAGATEVD and control peptide 2, WWWQTFDAR; and fusion peptide, DRGWGNGSGLFGKGG. The control peptides contained sequences unrelated to the fusion sequence and the fusion peptide contained the entire fusion sequence with a Ser substitution for Cys. In a direct binding assay, each of the oligopeptides was coated on a 96-well microtiter plate at 5 μg/well in 0.1 M carbonate buffer, pH 9.6. After washing

with PBS containing 0.05% Tween 20 and then blocking with PBS containing 3% bovine serum albumin (BSA), Fab 1A5 in PBS containing 1% BSA was added. Fab 1A5 bound to the oligopeptides was detected using goat anti-human IgG-alkaline phosphatase (Sigma). In a competition binding assay, purified Fab 1A5 at 0.05 μg/ml was pre-incubated with each of the oligopeptides in serial dilution at 37 C for 2 h. The reaction mixture was added to the wells of a microtiter plate coated with 25 μl of DENV-2 at 10^5 pfu/ml in PBS plus 1% BSA. Fab 1A5 bound to DENV-2 was detected as described.

Plaque morphology and growth analysis. Vero cells in a 6-well plate were infected with parental DENV-2 NGB, DENV-2 NGC, or an antigenic variant and overlaid with medium containing 1% gum tragacanth. After incubation at 37 C for 5 days, viral plaques were visualized by immuno-staining. The diameter of 20 plaques from each virus was measured on a digital image using Adobe Photoshop. For growth analysis, confluent monolayers of Vero cells or C6/36 cells in a 24-well plate were infected with each virus at 0.01 multiplicity of infection (moi) in duplicate. Infected Vero cells were incubated at 37 C and C6/36 cells at 32 C, and the culture medium was collected daily for 7 days. The virus sample was clarified by centrifugation and the titer determined by focus assay on Vero cells.

Mouse neurovirulence. Neurovirulence of parental DENV-2 NGB and its antigenic variants was evaluated in outbred Swiss mice. Three-day-old suckling mice, in groups of 8 to 11, were inoculated by the intracranial (ic) route with 100, 10, or 1 pfu of each virus in 20 μl MEM containing 0.25% human serum albumin. Inoculated mice were observed for symptoms of encephalitis, including ruffled hair, hunched back, paralysis and death. Paralyzed, morid-bound mice were euthanized and scored during the four-week observation period. Student's *t*-Test was used to compare the LD_{50} in pfu between parental DENV-2 and its antigenic variants.

Fusion activity assay. Fusion-from-within (FFWI) assays were performed for the DENV-2 parent and its antigenic variants as described (38). C6/36 cell monolayers in a 24-well plate were infected with each virus at 0.2 moi in MEM plus 10% FBS, buffered

with 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) at pH 7.7 and incubated at 32 C. Four to five days after infection, the infected cell monolayer was rinsed once with PBS and fusion medium (MEM plus 20 mM HEPES for pH 7.0 to 7.8 or 20 mM 2-morpholinoethanesulonic acid (MES) for pH 5.4 to 6.6 was added before incubation at 40 C for 2 hr. The infected cells were stained using the Diff-Quik Stain Set (Dade Behring) and examined for syncytium formation microscopically. The fusion index defined as (1- [number of cells/number of nuclei]) was calculated by counting 300 nuclei for each virus in at least five microscopic fields. The percentage of infected cells was determined by immunofluorescence assay using HMAF. Fusion inhibition by Fab 1A5 was performed as described (15). In brief, DENV-1 or DENV-2 infected C6/36 cells were incubated with Fab 1A5 at 37 C for 1 h prior to exposure to the low pH medium. Infected cells were also incubated with Mab 3H5 in parallel as the control.

Results

Selection of DENV-2 antigenic variants using Fab 1A5.

Mouse-adapted, neurovirulent DENV-2 NGB and DENV-2 NGC were used for selection of antigenic variants resistant to Fab 1A5 by neutralization *in vitro*. One DENV-2 NGB antigenic variant, designated as NGB-V1, was isolated after 8 cycles of neutralization and Vero cell passage. The $PRNT_{50}$ titer of NGB-V1 was 12.0 μg/ml, compared to that of parental DENV-2 NGB, which was 0.74 μg/ml (Fig. 1A). A second antigenic variant, designated NGB-V2, was isolated after 11 rounds of neutralization. NGB-V2 was completely resistant to neutralization by Fab 1A5 (> 70 μg/ml). In parallel, selection of DENV-2 NGC variants of Fab 1A5 was also performed to provide additional information. This effort yielded one antigenic variant, termed NGC-V2. The $PRNT_{50}$ titer of NGC-V2 was >70 μg/ml, compared to that of parental DENV-2 NGC, which was 0.89 μg/ml (Fig. 1B).

Sequence analysis of DENV-2 antigenic variants.

To map the Fab 1A5 epitope, the C-prM-E genes of antigenic variants NGB-V1, NGB-V2 and NGC-V2, and the parental viruses were sequenced. Variant NGB-V1 contained five nucleotide mutations in E, compared to the sequence of parental DENV-2

NGB (Table 1). Only the mutation at nucleotide 951 resulted in an amino acid substitution, Gln for His, at position 317 in E, whereas other nucleotide changes were silent mutations. The E sequence of variant NGB-V2 contained two nucleotide changes: a silent mutation of C to T at nucleotide 222, which was also present in NGB-V1, and a G to T mutation at position 317 that resulted in substitution of Val for Gly at position 106. Nucleotide changes were not found in the C-prM genes of variant NGB-V1 or NGB-V2. Variant NGC-V2 contained only a G to T change at nucleotide 317 in E that resulted in substitution of Val for Gly at position 106, identical to that found in NGB-V2. Fig. 2 shows alignment of the flavivirus E sequences surrounding Gly_{106} (panel A) and His_{317} (panel B). Gly_{106} is located within the 12-amino acid fusion peptide sequence (positions 98-109) that is nearly conserved among the arthropod-borne flaviviruses. His_{317} in E is also conserved among flaviviruses, although the surrounding sequences varied. In the 3-D structure, Gly_{106} is located in the cd loop at the tip of domain II and His_{317} is located between β-sheets A and B in domain III (Figs. 3A and 3B). Despite their locations in different domains, Gly_{106} and His_{317} of the opposite E monomer are spatially close, approximately 16 Å apart, calculated with Swiss Model (12).

Neutralization of DENV-2/DENV-4 chimeras by Fab 1A5.

Sequence analysis of antigenic variants indicated that Fab 1A5 appeared to recognize a novel epitope involving two closely spaced amino acids in different domains and from two interacting homodimers of DENV-2 E. The antigenic variants containing these mutations differed from the parent viruses in their Fab 1A5 neutralization titer. To provide additional evidence, we constructed DENV-2/DENV-4 chimeras composed of the parental DENV-2 NGB C-prM-E sequence or the variant C-prM-E sequence specifying the His_{317}-Gln or Gly_{106}-Val substitution present in NGB-V1 and NGB-V2, respectively, on the DENV-4 genetic background. As predicted, Fab 1A5 neutralized the chimeric DENV-2 (NGB-P)/DENV-4 at a $PRNT_{50}$ titer of 0.64 ug/ml, similar to that measured for parental DENV-2 NGB (data not shown). Substitution of Gly_{106}Val or His_{317}Gln in DENV-2 E of these chimeras conferred resistance to neutralization by Fab 1A5. The chimera containing Gly_{106}Val had a $PRNT_{50}$ titer of > 70 μg/ml and the chimera containing His_{317}Gln had a $PRNT_{50}$ titer of 31.7 μg/ml, similar to that measured

for NGB-V2 and NGB-V1, respectively.

Binding affinity of Fab 1A5 to antigenic variants.

To gain an insight into the neutralizing mechanism, the Fab 1A5 binding activity of the DENV-2 NGB parent virus and its variants was first analyzed by Western blotting. Mab 3H5, which had been shown to recognize an epitope at or near positions 383-385 of DENV-2 E (19), was used for comparison. Mab 3H5 reacted to the DENV-2 NGB parent, variant NGB-V1, NGB-V2, and each of the chimeras similarly. Under the same conditions, Fab 1A5 reacted with the DENV-2 NGB parent and variant NGB-V1, but not with variant NGB-V2 (Figs. 4A, top panel). Similarly, binding of Fab 1A5 to the DENV-2 NGB-V1/DENV-4 chimera, but not the DENV-2 NGB-V2/DEN4 chimera was observed (Fig. 4A, bottom panel).

An ELISA was performed to semi-quantify the binding affinity of Fab 1A5 for DENV-2 NGB and its two variants (Fig. 4B and Table 2). The apparent binding affinity *Kd* of Fab 1A5 for highly resistant variant NGB-V2 was the lowest among the three viruses. Thus, Gly_{106} represented a major determinant of the Fab 1A5 epitope on the DENV-2 E. On the other hand, the binding affinity of Fab 1A5 for variant NGB-V1 was not appreciably reduced, compared to that for the DENV-2 NGB parent. It is possible that His_{317} represented a minor determinant of the Fab 1A5 epitope and affected Fab 1A5 neutralization only indirectly.

Disulfide bridge dependency of the Fab 1A5 epitope.

In the DENV-2 E sequence, Fab 1A5 epitope determinant Gly_{106} is followed by Cys_{105}, which forms a disulfide bridge with Cys_{74}. It was of interest to provide data in support of the requirements of this and other disulfide bridges for functional integrity of the Fab 1A5 epitope. Treatment of DENV-2 NGB with β-mercaptoethanol abolished binding of Fab 1A5, as determined by Western blot analysis. Mab 3H5, which recognizes a conformational epitope on DENV-2 E also failed to bind DENV-2 NGB that was similarly treated (data not shown).

Reactivity of Fab 1A5 to an oligopeptide containing the fusion peptide sequence.

Two separate assays were performed to detect the reactivity of Fab 1A5 with oligopeptides bearing the fusion peptide sequence or unrelated sequences. Binding of Fab 1A5 to each of these oligopeptides (see Materials and Methods) immobilized on wells of a microtiter plate was not detected (data not shown). Competition binding was then performed in which Fab 1A5 was allowed to bind the individual oligopeptides in solution prior to testing for binding to DENV-2. The result in Fig. 5 indicates that binding of Fab 1A5 to DENV-2 was competed by the fusion peptide sequence, whereas each of the two control peptides containing unrelated sequences failed to compete, or only poorly. One interpretation of this result is that the oligopeptide in solution was able to assume the conformation that is required for binding to Fab 1A5.

Growth analysis of DENV2 NGB antigenic variants.

Four days after infection of Vero cells, parental DENV-2 NGB, DENV-2 NGC and variant NGBV-1 containing $His_{317}Gln$ substitution produced plaques similar in size, averaging 1.2 ± 0.2, 1.3 ± 0.1, and 1.1 ± 0.2 mm, respectively. Under the same conditions, variant NGB-V2 and NGC-V2 containing the $Gly_{106}Val$ substitution produced plaques of 0.4 ± 0.1 and 0.6 ± 0.1 mm, respectively, appreciably smaller than their parental virus. The growth kinetics of variant NGB-V1 and its parental virus were similar in C6/36 cells and in Vero cells (Figs. 6A and 6B). On the other hand, variant NGB-V2 consistently yielded a titer ten-fold lower than its parental virus in C6/36 cells and in Vero cells during the log-phase period, i.e., at 3, 4 and 5 days after infection. Similarly, $Gly_{106}Val$ substitution reduced replication of DENV-2/DENV-4 chimeras in C6/36 and Vero cells (Figs. 6C and 6D). The chimera containing $His_{317}Gln$ replicated to a level that was comparable to that of NGB-V1 in C6/36 cells. For reasons not understood, the chimeras containing $His_{317}Gln$ failed to replicate in Vero cells. Thus, Fab 1A5 selected antigenic variants that were attenuated, or at least, similar to the parental virus for growth in mammalian or insect cells.

Mouse neurovirulence of DENV-2 antigenic variants.

Mouse neurovirulence of the DENV-2 NGB antigenic variants was evaluated by intracranial inoculation of three-day-old outbred Swiss mice. Mice infected with the

DENV-2 NGB parent developed symptoms of encephalitis and eventually succumbed to infection (I. Tokimatsu, unpublished observations). Table 3 shows that the LD_{50} of variant NGB-V1 was 8.9 pfu, not significantly different from the LD_{50} of 4.5 pfu calculated for the parental virus. The LD_{50} of variant NGB-V2 at 16.4 pfu was significantly lower than that of the parental virus, indicating that the variant containing $Gly_{106}Val$ substitution was attenuated.

Fusion activity of DENV-2 antigenic variants.

Since the mutation site of variant NGB-V2 was mapped within the flavivirus-conserved fusion peptide loop, the attenuating phenotype of the variant might be associated with alteration of membrane fusion. Initially, the fusion activity of the DENV-2 NGB parent and its variants was examined on infected C6/36 cells. Syncytium formation of the cell monolayer was evident 2 days after infection with parental DENV-2 NGB. At 4 to 5 days after infection, cells of the entire monolayer formed syncytia and the cytopathic effect was extensive. In contrast, formation of syncytium was not observed on cells infected with either NGB-V1 or NGB-V2 under the same conditions, and the cytopathic effect was not seen till 7 days of infection. Reduced fusion of C6/36 cells infected with the DENV-2/DENV-4 chimeras containing the amino acid substitution present in NGB-V1 or NBG-V2 was also evident, compared to cells infected with the chimera containing the parental sequence (data not shown).

We also studied fusion of C6/36 cells infected with DENV-2 NGB and its antigenic variants at various pH's using the FFWI assay. Little or no fusion was observed at pH 7.0, 7.4 and 7.8. At pH 6.8, approximately 84% of the cells infected with parental DENV-2 formed syncytia. In contrast, 37% of cells infected with variant NGB-V1 and 46% of cells infected with variant NGB-V2 formed syncytia. Fig. 7 shows the fusion activity in terms of the index (FI) for the DENV-2 NGB parent and its variants determined at various pH's. Accordingly, the DENV-2 NGB parent had a pH threshold for 50% maximum fusion activity (FI = 0.5) at pH 6.77; variant NGB-V1 at pH 6.55; and variant NGB-V2 at pH 6.41.

Neutralizing activity of Fab 1A5 against DENV-4 mutants containing $Gly_{106}Val$ or

$Leu_{107}Phe$ substitution in the fusion loop.

Alignment of the flavivirus fusion sequences indicates JEV SA 14-14-2 contains a substitution of Phe for Leu at position 107, and LGTV a His substituting for Gly at position 104 (Fig. 2). The neutralizing activity of Fab 1A5 against JEV SA 14-14-2 and LGTV was the lowest among the flaviviruses tested (10). We questioned if substitution of $Leu_{107}Phe$ or $Gly_{104}His$ contributed to the resistance of these viruses to Fab 1A5 neutralization. The question whether Gly_{106} represented a determinant of the Fab 1A5 epitope on DENV-4 E was also raised.

To address the above questions in aggregate, full-length DENV-4 DNA was used to construct mutants containing various substitutions in the fusion peptide for analysis. DENV-4 mutants containing either $Gly_{106}Val$ or $Leu_{107}Phe$ were successfully constructed, however, a DENV-4 mutant containing the $Gly_{104}His$ substitution was apparently not viable. Fig. 8A shows the binding of Fab 1A5 to the DENV-4 parent and mutants containing a $Leu_{107}Phe$ or $Gly_{106}Val$ substitution. Fab 1A5 for the DENV-4 mutant containing $Gly_{106}Val$ had a binding affinity of Kd >40 nM, significantly reduced as compared to the DENV-4 parent (Kd=0.65 nM; $P < 0.0001$). Similarly, substitution of $Leu_{107}Phe$ in DENV-4 lowered the binding affinity of Fab 1A5 to a Kd at 3.07 ± 0.27 nM, $p < 0.001$. Fig. 8B presents Fab 1A5 neutralization of the DENV-4 parent and mutants. The neutralizing titer of Fab1A5 against each of the DENV-4 mutants was greatly reduced compared to that against DENV-4. These observations suggest that both Gly_{106} and Leu_{107} are Fab 1A5 epitope determinants on DENV-4 E.

Discussion

Epitope analysis of chimpanzee Fab 1A5 was performed to gain an insight into the binding site on the E protein responsible for the flavivirus cross-reactivity and the mechanism for cross-neutralization of dengue viruses and other flaviviruses. Our current understanding of the flavivirus E antigenic structure and functional activities has been obtained from studies using mouse monoclonal antibodies against these viruses. The present study represents the first analysis of any primate-derived monoclonal antibodies against an arthropod-borne flavivirus.

Analysis of mutations in the C-prM-E region of DENV-2 antigenic variants

established that Fab 1A5 recognized a novel epitope on DENV-2 E protein that is defined by two closely spaced amino acids from the two neighboring E subunits of the homodimer. Gly_{106} at the tip of the flavivirus-conserved fusion loop in domain II represents a major determinant of the Fab 1A5 epitope on DENV-2 E. DENV-2 variants and chimeras containing a Val substitution at this position failed to bind Fab 1A5 and these viruses were completely resistant to Fab 1A5. Substitution of Val for Gly_{106} is a non-conserved amino acid change, resulting in an increase of the mean hydrophobicity in the Cys-Gly-Leu tripeptide segment (positions 105-107) from 1.69 for parental DENV-2 to 2.69 for the variant.

Evidence suggests that His_{317} in domain III from the adjacent E monomer represents another determinant of the Fab 1A5 epitope. First, the DENV-2 antigenic variant or DENV-2/DENV-4 chimera containing His_{317}Glu substitution was partially resistant to neutralization by Fab 1A5. Second, in the 3D structure both Gly_{106} and His_{317} were spatially close, calculated to be 16.2 Å apart, well within the distance for antibody binding. Substitution of His_{317}Gln in DENV-2 E did not significantly alter the hydrophobicity of this region and had little, if any, effect on the binding of Fab 1A5l. Thus, the mechanism for the involvement of this amino acid in Fab 1A5 neutralization is more complicated. Mutations in flavivirus or other virus escape mutants have been localized outside the antibody binding sites (3, 20, 49). Such mutations might affect epitopes for antibody neutralization indirectly. Thus, His_{317} might play a lesser role than did Gly_{106} in the interactions with Fab 1A5. It can be assumed that the existence of the Fab 1A5 epitope requires inter-domain interactions occurring either before or following the conformational shift from dimeric to trimeric E. Monoclonal antibodies that react with an epitope located in the interface between two adjacent subunits have been described for the influenza neuraminidase (43).

There is ample evidence indicating that the flavivirus fusion loop directly participates in low pH-induced membrane fusion believed to take place following viral entry. The low pH-triggered membrane fusion involves the dissociation of the flavivirus E dimer and subsequent formation of the E trimer (2, 14, 44, 45). Recent structural studies indicate that such a conformational switch allows the fusion loop in the interface between domain I and III of the dimeric E to become exposed, realigned, and made available for fusion

interaction with the endosomal membrane (6, 32). Using a liposomal membrane model, it has been shown that recombinant subviral particles of TBEV prM and E containing a substitution of Asp or Phe for Leu_{107} in the fusion peptide sequence were defective for membrane fusion (1). It has been reported that several monoclonal antibodies recognizing the domain A epitope (domain II) lost and regained their reactivities to TBEV upon lowering the pH (13). It is possible that these monoclonal antibodies recognize a site in E that involves membrane fusion. DENV-2 monoclonal antibodies capable of blocking membrane fusion of DENV-2 infected cells have also been shown to react with a tryptic peptide fragment consisting of the first 120 amino acids of E, although the epitope determinants of these antibodies have not been precisely mapped (41).

The current study with monoclonal antibody Fab 1A5 has provided additional data supporting the involvement of the fusion loop in membrane fusion. First, a major epitope determinant of Fab 1A5 maps at Gly_{106} within the fusion peptide loop. Second, Fab 1A5 inhibited membrane fusion as demonstrated by reduced syncytium formation of DENV-2- and DENV-1-infected C6/36 cells. Third, the DENV-2 antigenic variants of Fab 1A5 that contained a Gly_{106}Val substitution lowered the pH threshold for fusion of infected C6/36 cells. Binding of Fab 1A5 to DENV-2 and DENV-1 E probably inhibits the low-pH induced conformational change of E, rendering the subsequent fusion step defective following viral entry.

Structural analysis of TBEV E indicates that His_{323} (His_{317} in DENV-2) also plays a role in the pH-induced transition of the E homodimer to become a homotrimer (6). The flavivirus-conserved His_{323} of domain III forms a hydrogen bond with another flavivirus-conserved residue in domain I to stabilize the inter-domain contact between two E monomers. These investigators further suggest that protonation of this His residue serves to facilitate dissociation of the E homodimers at a low pH. In trimer E, domain III packs the interface of domain I and domain II. His_{323} also participates in the interaction. Our result showing that DENV-2 variant NGB-V1 containing a His_{317}Gln substitution has a lower fusion threshold for membrane fusion than the parental DENV-2 provides evidence for the involvement of His_{317} in membrane fusion.

Conservation of the fusion peptide sequence among flaviviruses suggests that the fusion peptide plays a central role in viral replication and variants containing mutations in

this region would be at an evolutionary disadvantage. Examination of the sequences in this region among flaviviruses indicates that JEV SA-14-14-2 contains a $Leu_{107}Phe$ substitution, compared to the sequence of the parental JEV SA-14 strain (35). JEV strain SA-4-14-2 was attenuated and has been used widely as a live vaccine in China. It remains to be determined whether the $Leu_{107}Phe$ mutation was principally responsible for the attenuating phenotype, as there are other mutations in the attenuated JEV (35). The virulent parental JEV SA-14 strain has a fusion peptide sequence identical to that of the dengue viruses and WNV. Importantly, other BSL-3 flaviviruses of medical importance, including SLEV and YFV, also have fusion peptides that share this exact sequence (Fig. 2). It will be interesting to determine if these viruses are also neutralized by Fab 1A5. However, it is clear that mutations in or near the flavivirus fusion peptide are not the sole determinants of attenuation since the relatively attenuated LGTV and the virulent TBEV have identical sequence in the two regions studied here (27, 28), as do the attenuated YFV 17D strain and its virulent parent Asibi virus (16, 40).

Compared to the parental virus, the DENV-2 antigenic variant containing $Gly_{106}Val$ replicated less favorably in cultured simian Vero and mosquito cells *in vitro* and exhibited reduced neurovirulence in mice. Naturally occurring dengue virus variants, if they should ever emerge in the presence of the Fab 1A5 antibody, would be expected to be attenuated or at least similar to the parental virus. In this regard, Fab 1A5 or its humanized antibody appears to have an added safety feature for use in passive immunization against dengue and other flaviviruses.

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Figure Legends

Figure 1. Neutralization of DENV-2 parental viruses and their variants using Fab 1A5. (A) NGB-P, DENV-2 NGB parent; DENV-2 variant NGB-V1; DENV2 variant NGB-V2. (B) NGC-P, DENV-2 NGC parent; DENV-2 variant NGC-V2.

Figure 2. Alignment of amino acid sequences among flaviviruses.
(A) shows the sequences surrounding Val_{106} found in DENV-2 variants NGB-V2 and NGC-V2. The fusion sequence (loop) between c and d β-sheets is underlined. (B) shows the sequences surrounding Gln_{317} present in DENV-2 variant NGB-V1. The sequence between A and B β-strands is underlined. The references of the flavivirus sequences are as follows: DENV-1 (29); DENV-2 (17); DENV-3 (36); DENV-4 (50); WNV (24, 48); SLEV (47); JEV JaOArS982 (46); JEV SA-4-14-2 (35); YFV 17D (40); YFV Asibi (16); LGTV (28); TBEV (27).

Figure 3. Localization of Fab 1A5 epitope determinants in 3-D structure of DENV-2 E. (A) shows positions of Gly_{106} and His_{317} as viewed from the top of the structure using the published coordinates (31). (B) shows the expanded area of the insert above.

Figure 4. Reactivity of Fab 1A5 to DENV-2 NGB parent and its antigenic variants. Panel A (top) shows binding of control Mab 3H5 (which does not bind to the fusion peptide) to various viruses by Western blot analysis as control. Gel lanes; 1, DENV-2, NGB parent; 2, DENV-2 NGB-V1; 3, DENV-2 NGB-V2; 4, NGB-parent/DENV-4 chimera; 5, NGB V1/DENV-4 chimera; 6, NGB-V2/DENV-4 chimera. Panel A (bottom) shows binding of Fab 1A5 to the viruses listed above by Western blot analysis. Panel B shows binding of Fab 1A5 to the DENV-2 NGB parent and its antigenic variants by ELISA.

Figure 5. Inhibition of Fab 1A5 binding to DENV-2 by a fusion peptide.
In the binding competition assay, Fab 1A5 was mixed with serial dilutions of an oligopeptide containing the entire fusion peptide sequence or a control peptide with an unrelated sequence (see Materials and Methods). The mixture was tested for binding to

DENV-2 coated on an ELISA plate.

Figure 6. Growth analysis of antigenic variants, chimeras, and the DENV-2 NGB parent in cultured cells. Each of the viruses was analyzed for growth in C6/36 cells (A) and in Vero cells (B). Chimeras that contained C-prM-E of the parental NGB, variant NGB V-1 or NGB V-2 on the DENV-4 background were similarly analyzed for growth in C6/36 cells (C) and in Vero cells (D).

Figure 7. Fusion activity of DENV-2 NGB parent or its variants. Fusion from within assay was performed on C6/36 cells infected with each of the viruses. The fusion activity at various pHs was detected by syncytium formation. The fusion index was calculated to determine the pH threshold for each virus.

Figure 8. Binding activity of Fab 1A5 to DENV-4 parent and DENV-4 mutants containing a substitution of $Gly_{106}Val$ or $Leu_{107}Phe$ in the fusion loop (panel A) and neutralizing activity of Fab 1A5 against these viruses (panel B).

Table 1. Nucleotide and amino acid changes in the E proteins of antigenic variants as compared to their parental viruses.

Variant	Nucleotide change	Amino acid change	Domain
NGB-V1[1]	$^{222}C \rightarrow T$	No	
	$^{402}T \rightarrow C$	No	
	$^{468}A \rightarrow G$	No	
	$^{526}A \rightarrow G$	No	
	$^{951}T \rightarrow A$	$^{317}His \rightarrow Gln$	III
	$^{222}C \rightarrow T$	No	
NGB-V2[1]	$^{222}C \rightarrow T$	No	
	$^{317}G \rightarrow T$	$^{106}Gly \rightarrow Val$	II
NGC-V2[2]	$^{317}G \rightarrow T$	$^{106}Gly \rightarrow Val$	II

[1] No amino acid changes were found in the C-PreM region.
[2] A substitution of Ala for Thr at position 280, the last amino acid of prM, was found.

Table 2. Apparent binding affinities of Fab 1A5 for parental DENV-2 NGB and its variants.

DENV- 2	*Kd* (nM)	Affinity Reduction (fold)
NGB-P	0.47 ± 0.18	
NGB-V1	0.75 ± 0.31	1.60
NGB-V2	37.75 ± 1.11	80.32

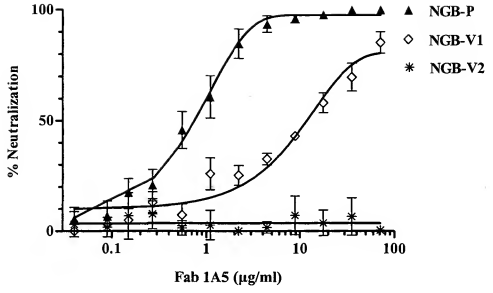
NGB-P indicates parental DENV-2 NGB.

Table 3. Neurovirulence of parental DENV-2 NGB and its variants following ic inoculation in suckling Swiss mice.

Virus	Mortality of mice after ic inoculation with the indicated virus at pfu of			Mean LD_{50} ± SE (pfu)
	100	10	1	
NGB-P	20/20 (100%)	19/21 (90.5%)	3/10 (30%)	4.52 ± 0.07
NGB-V1	19/20 (95%)	14/20 (70%)	2/10 (20%)	8.9 ± 3.6*
NGB-V2	18/18 (100%)	9/19 (47.4%)	2/10 (20%)	16.4 ± 0.28**

NGB-P indicates parental DENV-2 NGB.
*P=0.23; **P= 0.0065.

A


100
50
0
% Neutralization
0.1
1
10
100
Fab 1A5 (µg/ml)
NGB-P
NGB-V1
NGB-V2

B

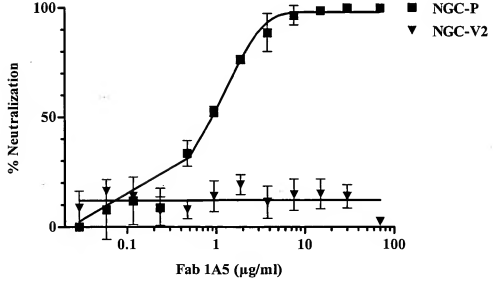
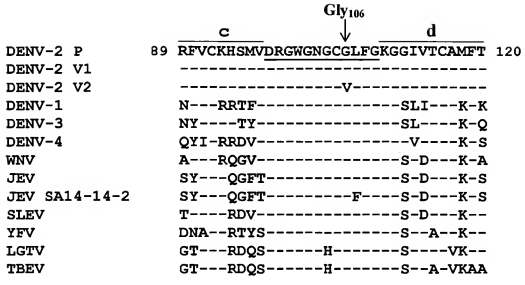

100
50
0
% Neutralization
0.1
1
10
100
Fab 1A5 (µg/ml)
NGC-P
NGC-V2

FIG. 1

A


Gly106
c
d
DENV-2 P 89 RFVCKHSMVDRGWGNGCGLFGKGGIVTCAMFT 120
DENV-2 V1 ----------
DENV-2 V2 ----------V----------
DENV-1 N---RRTF----------SLI---K-K
DENV-3 NY----TY----------SL----K-Q
DENV-4 QYI-RRDV----------V----K-S
WNV A---RQGV----------S-D---K-A
JEV SY---QGFT----------S-D---K-S
JEV SA14-14-2 SY---QGFT---------F----S-D---K-S
SLEV T----RDV----------S-D---K--
YFV DNA--RTYS----------S--A--K--
LGTV GT---RDQS------H-------S----VK--
TBEV GT---RDQS------H-------S--A-VKAA

B

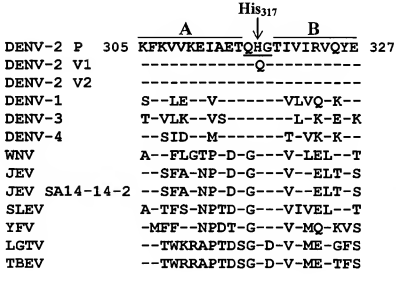
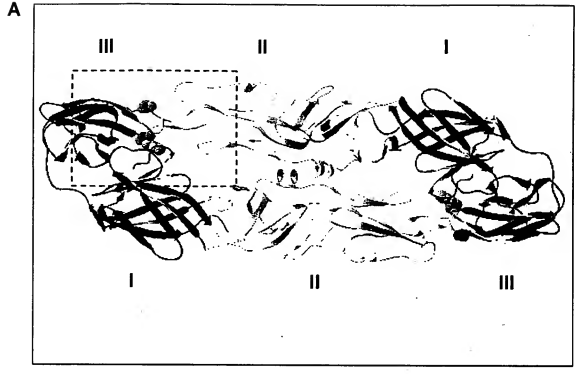

His317
A
B
DENV-2 P 305 KFKVVKEIAETQHGTIVIRVQYE 327
DENV-2 V1 ----------Q----------
DENV-2 V2 ----------
DENV-1 S--LE--V-------VLVQ-K--
DENV-3 T-VLK--VS-------L-K-E-K
DENV-4 --SID--M-------T-VK-K--
WNV A--FLGTP-D-G---V-LEL--T
JEV --SFA-NP-D-G---V--ELT-S
JEV SA14-14-2 --SFA-NP-D-G---V--ELT-S
SLEV A-TFS-NPTD-G---VIVEL--T
YFV -MFF--NPDT-G---V-MQ-KVS
LGTV --TWKRAPTDSG-D-V-ME-GFS
TBEV --TWRRAPTDSG-D-V-ME-TFS

FIG. 2



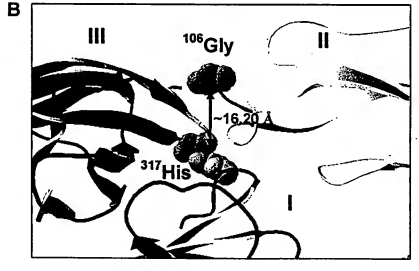
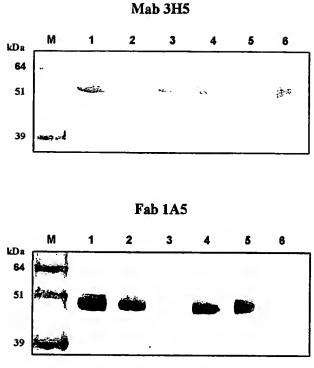


FIG. 3

A


Mab 3H5
kDa
M
1
2
3
4
5
6
64
51
39
Fab 1A5
kDa
M
1
2
3
4
5
6
64
51
39

B

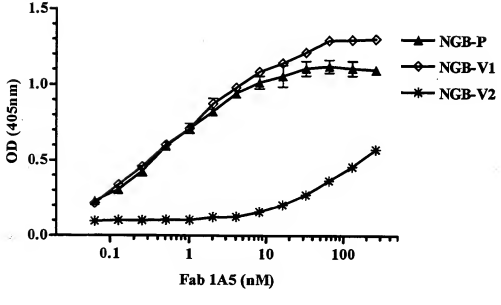

1.5
1.0
0.5
0.0
OD (405nm)
0.1
1
10
100
Fab 1A5 (nM)
NGB-P
NGB-V1
NGB-V2

FIG. 4

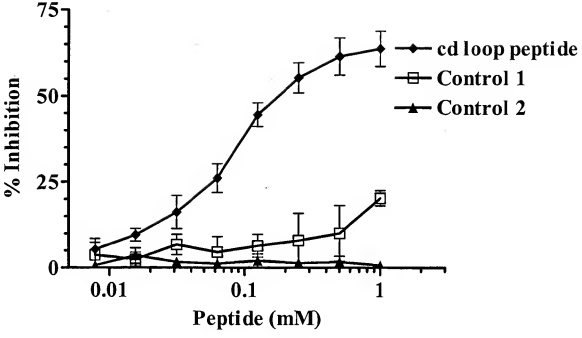

75
50
25
0
% Inhibition
0.01
0.1
1
Peptide (mM)
cd loop peptide
Control 1
Control 2

FIG. 5

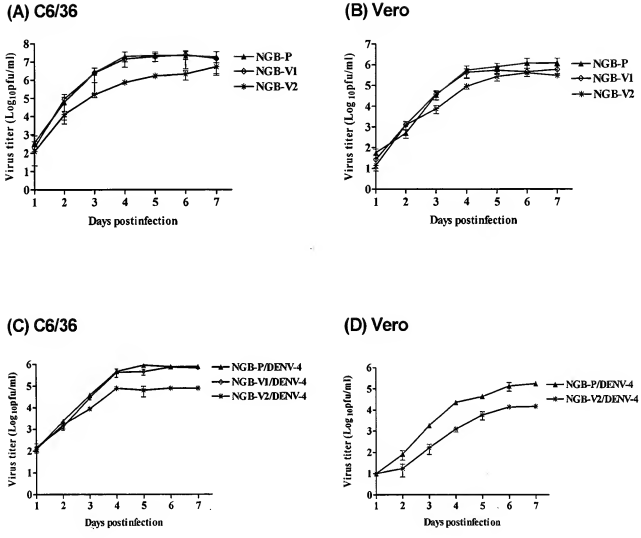

(A) C6/36
Virus titer (Log10pfu/ml)
Days postinfection
NGB-P
NGB-V1
NGB-V2
(B) Vero
Virus titer (Log 10pfu/ml)
Days postinfection
NGB-P
NGB-V1
NGB-V2
(C) C6/36
Virus titer (Log10pfu/ml)
Days postinfection
NGB-P/DENV-4
NGB-V1/DENV-4
NGB-V2/DENV-4
(D) Vero
Virus titer (Log 10pfu/ml)
Days postinfection
NGB-P/DENV-4
NGB-V2/DENV-4

FIG. 6

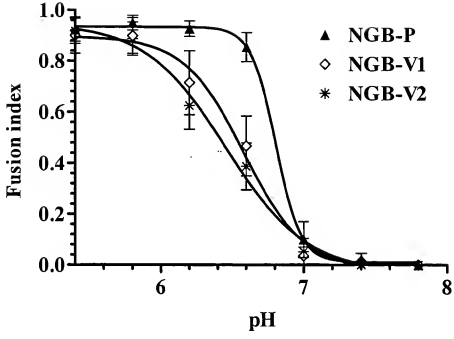
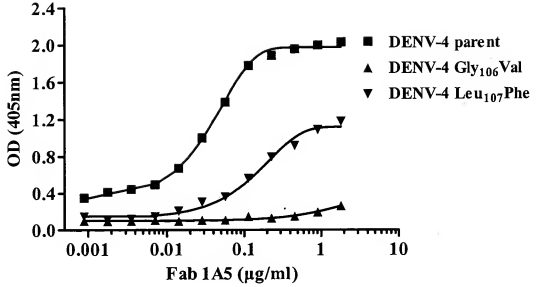


FIG. 7

A


2.4
2.0
1.6
1.2
0.8
0.4
0.0
OD (405nm)
0.001
0.01
0.1
1
10
Fab 1A5 (μg/ml)
DENV-4 parent
DENV-4 Gly106Val
DENV-4 Leu107Phe

B

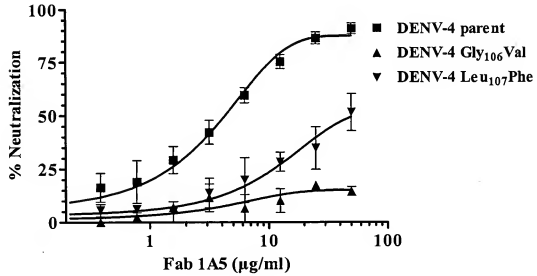

100
75
50
25
0
% Neutralization
1
10
100
Fab 1A5 (μg/ml)
DENV-4 parent
DENV-4 Gly106Val
DENV-4 Leu107Phe

FIG. 8